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Comparative Studies of the Mutagenicity of Environmental Tobacco Smoke From Cigarettes That Burn or Primarily Heat Tobacco

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The mutagenicity of particulate matter concentrated from environmental tobacco smoke (ETS) from a prototype cigarette that primarily heats tobacco was compared to that of four popular commercially available cigarettes that burn tobacco. ETS was generated by six individuals simultaneously smoking 1 cigarette each in a 20-min time period in a 45 m^3 environmental chamber operated in the static mode (without ventilation). Respirable suspended particles (RSP) were collected on polytetrafluoroethylene (PTFE) filters at a flow rate of 3 LPM for 120 min. Less ETS-RSP (86–90%) was emitted by the prototype tobacco-heating cigarette than by the tobacco-burning cigarettes. RSP was extracted from the filters by sequential sonication in acetone and dichloromethane. The acetone extract was dried under nitrogen and the dichloromethane filtrate was added and then dried to obtain ETS-RSP for testing. Mutagenicity was assessed in the microsuspension modification of the Ames Salmonella/microsome assay with strains TA98 and YG1024 in the presence of 5% S9 metabolic activation. The results show that the mutagenic activity of RSP from the prototype cigarette was reduced by 75–83% on a per-mg basis when compared to the commercially available cigarettes and was reduced by 96–98% when calculated as revertants/m^3 air under identical smoking conditions. Environ. Mol. Mutagen. 31:169–175, 1998 © 1998 Wiley-Liss, Inc.

Key words: Ames assay; environmental tobacco smoke; mutagenicity assay

INTRODUCTION

Environmental tobacco smoke (ETS) consists of a mixture of aged and diluted sidestream smoke emitted from the burning end of a cigarette, combined with aged and diluted mainstream smoke exhaled by the smoker. Particulate matter concentrated from sidestream smoke or ETS has been reported to be mutagenic in the Salmonella/mammalian microsome (Ames) assay [Löfroth and Lazardis, 1986; Claxton et al., 1989; Nardini et al., 1994]. R.J. Reynolds Tobacco Company has developed a new cigarette (TOB-HT) which primarily heats tobacco and burns only a small amount of tobacco (Fig. 1). The TOB-HT design consists of tobacco rolled in paper with a standard cellulose acetate filter. Combustion is contained within a small portion of the cigarette at the lit end, i.e., a carbon heat source. During smoking, tobacco flavors and added glycerin are vaporized from the heated tobacco rod to form the smoke aerosol. This design greatly reduces the amount of sidestream smoke produced by the lit end and the particulate matter in the mainstream smoke is approximately 80% glycerin and water compared to 80% particulate matter (including water and glycerin) in a typical cigarette. Taken together, these design changes would be expected to reduce both the quantity and the mutagenicity of the ETS particulate matter from this cigarette. The objective of the current study was to test this hypothesis.

The biological activity of mainstream smoke condensate from this new cigarette has been evaluated in an in vitro toxicology battery including the Ames Salmonella mutagenicity assay, sister chromatid exchange, chromosome aberration, and cytotoxicity in Chinese hamster ovary cells. In each of these assays the biological activity of smoke condensate from the TOB-HT cigarette is significantly reduced compared to tobacco-burning cigarettes [Bombick et al., in press]. In addition, a four-week study in smokers determined that when smokers switch to tobacco-heating cigarettes they void urine which is significantly less mutagenic than urine voided when smoking their usual brand of cigarettes [Smith et al., 1996].

The objective of this study was to compare the mutagenicity of concentrated ETS respirable suspended particles (RSP) from the TOB-HT cigarette to that of four commercially available cigarettes representing full flavor (≥14 mg "tar"), full flavor light (7–14 mg "tar"), and ultra-

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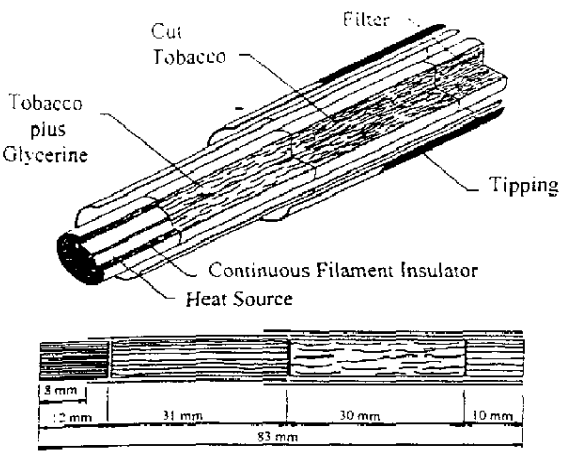

Fig. 1. Schematic of a cigarette that primarily heats tobacco.

low (<7 mg "tar") cigarettes in the microsuspension modification of the Ames Salmonella/microsome assay.

MATERIALS AND METHODS

Cigarettes

The TOB-HT prototype cigarettes were obtained from R.J. Reynolds; the four commercially available cigarettes were purchased at retail. The cigarettes tested are described in Table I. The cigarettes were characterized as full flavor (≥14 mg "tar"), full flavor light (7–14 mg "tar"), or ultra-low (<7 mg "tar").

Environmental Chamber

Collection of RSP for the assay was carried out in a [illegible] environment test chamber [Nelson et al., in press]. The surfaces of the chamber are stainless steel (floor, recirculation [illegible] on polyester powder coat over steel (walls and ceiling). [illegible] relative humidity, and ventilation can be precisely [illegible] chamber. Chamber supply air is dehumidified and then [illegible] charcoal and HEPA filters.

In order to generate an ETS concentration high enough to collect sufficient RSP for analysis, the chamber was operated in [illegible] mode (no ventilation or recirculation). To achieve the target [illegible] 24°C and relative humidity of 50%, the chamber temperature [illegible] humidity were regulated at 20°C and 57.5% RH prior to [illegible] each run.

Smoke Generation

To obtain representative results, human smokers generated ETS in the chamber. In this way, true ETS (a combination of both aged and diluted sidestream and exhaled mainstream smoke) was introduced in the chamber.

Whenever possible, the smokers smoked their normal brand/style in an experiment. For example, if an 85-mm box style of a particular brand of full-flavor cigarette was smoked in the chamber, then smokers who normally smoked that exact brand style were recruited to generate ETS. For TOB-HT cigarettes, smokers received product one week in advance of the test and substituted it for their normal brand so that they could become accustomed to the cigarette.

TABLE I. Description of Cigarettes

Cigarette	Description
TOB-HT[a]	Test cigarette which primarily heats tobacco
FF[b]	Full-flavor 85 mm
FFLT[b]	Full-flavor light 85 mm
100S[b]	Full-flavor light 100 mm
ULT[b]	Ultra-low tar 100 mm

[a]Prototype cigarette obtained from R.J. Reynolds Tobacco Co., Winston-Salem, N.C.
[b]Purchased at retail.

To ensure sufficient mass of sample for analysis, particularly with the TOB-HT cigarette, very high levels of ETS concentrations were necessary. To generate the ETS levels, six smokers entered the chamber. Following a 10-min background collection period, the smokers each lit one cigarette. They then smoked the cigarette ad libitum until the cigarette was consumed (to a line 3 mm from the overwrap for commercially available cigarettes and no further smoke aerosol for TOB-HT). At 20 min, the smokers exited the chamber, which was then resealed. Sampling pumps for ETS-RSP samples started at this point. The sample collection time was 120 min. For each cigarette tested, five replicate chamber runs were performed.

Sample Collection

Samples for gravimetric RSP analysis were collected on preweighed 37 mm Fluoropore® (polytetrafluoroethylene) filters (Millipore, Milford, MA). An inertial impactor with a 2.5 μm cut point was used upstream of each filter. A sample flow rate of ~3.2 liters/min was used. Six filters for Ames analysis were collected during each chamber run when commercial cigarettes were smoked and ten were collected during blank and TOB-HT runs. At the completion of the experiment, filter weights were analyzed according to the method of Ogden et al. [1996]. Data were also checked to determine if there were any problems with the collection of particles on any specific filter (previous experience has shown that some filter cassettes may leak during sampling). Filters with RSP levels that differed by more than 20% from the mean RSP level for a given run were discarded. Following reweighing, all filters were stored in a freezer at −70°C until they were extracted.

Additional analyte concentrations were determined using both real-time and time-weighted average methodologies. Results are described elsewhere in greater detail [Nelson et al., in press]. Analytes reported here include measurement of total hydrocarbons with an FID analyzer (Combustion Engineering model 8401, Lewisberg, WV), CO (Model 48, Thermo Environmental, Franklin, MA), and oxides of nitrogen (Model 41H, Thermo Environmental, Franklin, MA). Aldehydes were collected on Sep-Pak DNPH-silica cartridges (Waters, Milford, MA) and determined by the method of Risner [1995]. Phenols were collected on a combination of one 37 mm, 0.80, or 0.45 μm pore size, mixed cellulose ester membrane filter (SKC, Eight Four, PA) and one silica gel cartridge (Waters, Milford, MA) in series and analyzed by the method of Risner [1993]. Nicotine and 3-ethynylpyridine were collected on XAD-4 (SKC, Eight Four, PA) sorbent tubes and analyzed by GC with thermionic detection [Ogden et al., 1996].

Extraction of ETS-RSP

Different solvents for the extraction of air particulates for mutagenicity assessment have been reported in the literature. Lewtas et al. [1987]

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TABLE II. Description of Bacterial Strains Compared

Bacterial strain	Description
TA98 *rfa, ΔuvrB, hisD3052,* pKM101	Ames strain sensitive to frameshift mutations
YG1021 TA98 with pYG216	TA98 derivative with plasmid-enhancing nitroreductase activity
YG1024 TA98 with pYG219	TA98 derivative with plasmid-enhancing *O*-acetyltransferase activity
TA100 *rfa, ΔuvrB, hisG46,* pKM101	Ames strain sensitive to basepair substitution
YG1026 TA100 with pYG216	TA100 derivative with plasmid-enhancing nitroreductase activity
YG1029 TA100 with pYG219	TA100 derivative with plasmid-enhancing *O*-acetyltransferase activity

TABLE III. ETS Concentrations of Selected Analytes From Blank, TOB-HT, and Four Commercially Available Cigarettes

ETS component	Blank	TOB-HT	FF	FFLT	100s	ULT
RSP	18 ± 4	181 ± 11	1458 ± 29	1345 ± 20	1706 ± 23	1184 ± 27
Acetaldehyde	13 ± 2	46 ± 1	313 ± 13	301 ± 13	384 ± 20	312 ± 8
FID response (ppm)	0.15 ± 0.04	0.47 ± 0.04	2.6 ± 0.2	2.6 ± 0.2	3.0 ± 0.2	2.8 ± 0.1
NO_x (ppb)	7 ± 1	24 ± 1	241 ± 10	233 ± 5	268 ± 6	250 ± 5
3-Ethenylpyridine	0.36 ± 0.06	0.56 ± 0.06	25 ± 1	28 ± 1	28 ± 2	32 ± 4
Nicotine	2.3 ± 0.4	4.3 ± 0.6	54 ± 6	63 ± 6	58 ± 9	51 ± 9
Phenol	3.6 ± 0.4	4.0 ± 0.3	17.4 ± 0.6	16.7 ± 0.2	20.0 ± 0.2	16.8 ± 0.2
CO (ppm)	0.17 ± 0.01	5.20 ± 0.06	6.5 ± 0.1	6.2 ± 0.1	7.9 ± 0.1	5.9 ± 0.1

All units are μg/m³ except as noted, followed by standard errors.

and Kado et al. [1991] used dichloromethane for extraction of airborne mutagens. Morin et al. [1987] conducted a study comparing the extraction efficiency of dichloromethane, acetone, and methanol, and obtained the highest levels of mutagenic activity with ultrasonic agitation and acetone extraction. Lee et al. [1991] concluded that acetone was most effective in extraction of mutagens from airborne particulate samples; Nielsen [1992] reported that acetone may extract compounds from ambient air particles that dichloromethane may not extract. Krishna et al. [1983] reported that sequential extraction with acetone followed by dichloromethane gave a better mutagenic response than acetone alone. Due to these literature uncertainties, we conducted preliminary studies to determine the most efficient method for extraction of ETS-RSP. ETS-RSP from full flavor (FF) commercial tobacco-burning cigarettes was collected on filters and extracted with either acetone, dichloromethane, or sequential extraction in both acetone and dichloromethane. Mutagenicity was assessed in the microsuspension modification of the Ames assay using strains TA98 and YG1024. Extracts prepared by sonication of filters in acetone followed by sonication in dichloromethane had equal or greater mutagenicity than extracts prepared in either acetone or dichloromethane alone (data not shown), confirming the report of Krishna et al. [1983].

ETS-RSP was therefore extracted from the filters in this study by sequential sonication in acetone and dichloromethane. Filters were sonicated for 30 min in 20 ml of acetone. The filter was removed from the acetone and then sonicated for 30 min in 20 ml of dichloromethane. The acetone extract was evaporated to dryness under a stream of nitrogen at 45°C and the dichloromethane filtrate was added. The dichloromethane was then evaporated to dryness and the final RSP extract was resuspended in dimethyl sulfoxide at concentrations of 1 or 10 mg RSP/ml. Extracts were stored at −70°C in the dark until assayed.

Mutagenicity Analysis

Mutagenicity was assessed in the Salmonella/microsome assay [Maron and Ames, 1983] with the microsuspension modification described by Kado et al. [1983, 1986]. The liver homogenate used in S9 preparations was obtained from Mol-Tox (Annapolis, MD) and prepared according to Ames et al. [1975] from male Sprague-Dawley rats that were given a single 500 g/kg injection, i.p. of Aroclor 1254. RSP concentrates were added to 12 × 75 mm tubes. Concentrated tester strain (concentrated 5x in 0.02 M phosphate buffer from an overnight culture) and S-9 mix or phosphate buffer was added. Test tubes were incubated at 37°C while shaking at 200 RPM for 90 min. Two ml of top agar containing histidine (0.5 mM) and biotin (0.5 mM) was added and the mixture was poured onto minimal glucose plates. Plates were allowed to incubate at 37°C for up to 72 hr before revertants were scored. Concurrent negative (blank) and positive (2-anthramine, 0.025 μg/plate) controls were performed with all experiments. In addition, a standard cigarette smoke condensate from Kentucky Reference cigarette 1R4F was used as an internal assay control on each day of testing. Responses from 2-anthramine and 1R4F were within established guidelines for the laboratory (data not shown). All testing was conducted with triplicate plates at each dose level. A positive response was defined as a dose-dependent relationship with revertant number double the background at least one dose. Slope values (revertants per μg sample) were calculated by a linear point-rejection model [Bernstein et al., 1982].

Initial assays were conducted with FF RSP to determine the optimum test conditions for the study. RSP was assayed at concentrations of 2.5, 5, 7.5, 10, 15, and 20 μg per plate with 0, 1%, and 5% S9. RSP was assayed with both frameshift and base substitution strains TA98 and TA100 as well as with recently developed strains YG1021, YG1024, YG1026, and YG1029. The YG strains have inserted plasmids that confer elevated nitroreductase (YG1021, YG1026) or acetyltransferase (N-hydroxyarylamine *O*-acetyltransferase activity (YG1024, YG1029); the strains are described in Table II. These are strains which have been successfully used in airborne mutagenicity studies [Houk et al., 1992; Morris et al., 1995]. The additional acetyltransferase provides improved detection of aromatic amines, while the additional nitroreductase improves detection of nitroarenes [Einistö et al., 1991].

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TABLE IV. Reduction of Environmental Tobacco Smoke Components of TOB-HT Cigarettes Compared to Tobacco-Burning Cigarettes

ETS component	% Reduction of TOB-HT cigarette relative to tobacco-burning cigarettes
Respirable suspended particles	86–90
Total hydrocarbons	87–90
NO_x	93–94
Aldehydes	86–91
Phenols	>95
Nicotine and ethenylpyridine	96–99
CO	11–30

RESULTS

The concentrations of representative ETS constituents were determined in the environmental chamber following smoking of cigarettes. Background measurements showed only trace amounts of RSP prior to the initiation of smoking. The TOB-HT cigarette produced 86–90% less ETS-RSP than the four reference commercial cigarettes, and specific ETS components were also significantly reduced (Tables III, IV). With the exception of CO (reduced 11–30%) the average background-corrected reduction of all ETS components was at least 80%; the majority of measured chemical components were reduced by greater than 90% (Tables III, IV) [Nelson et al., in press].

In studies to select the most sensitive Salmonella tester strains for quantifying the mutagenicity of ETS-RSP, TA98, YG1024, and YG1026 showed positive responses (Fig. 2). YG1024 had the greatest sensitivity to mutagens in the concentrated ETS-RSP extracts. To optimize the S9 concentration, RSP was evaluated in all six strains in the presence of 0, 1, and 5% S9. Mutagenicity was greatest with 5% S9 in all six strains; data is shown with TA98 and YG1024 only in Figure 3.

Based on the results of these assays, the final study conditions were microsuspension modification of the Ames assay with 5% S9, strains TA98 and YG1024. RSP from the tobacco-burning cigarettes was tested up to 20 or 40 μg/plate in five separate assays; RSP from the TOB-HT cigarette was tested up to 40 μg/plate in three separate assays.

The mutagenicity of concentrated ETS-RSP from TOB-HT was compared to that of the four tobacco-burning cigarettes on both a revertant/mg (specific activity) and calculated revertants/cubic meter basis. The mutagenicity of ETS-RSP from TOB-HT cigarettes was reduced by 75–83% on a per-mg basis (Fig. 4) compared to the four commercially available cigarettes. The mutagenic activity of RSP from TOB-HT was reduced by 96–98% when calculated on a revertants/m^3 basis (Fig. 5) compared to the four commercially available cigarettes.

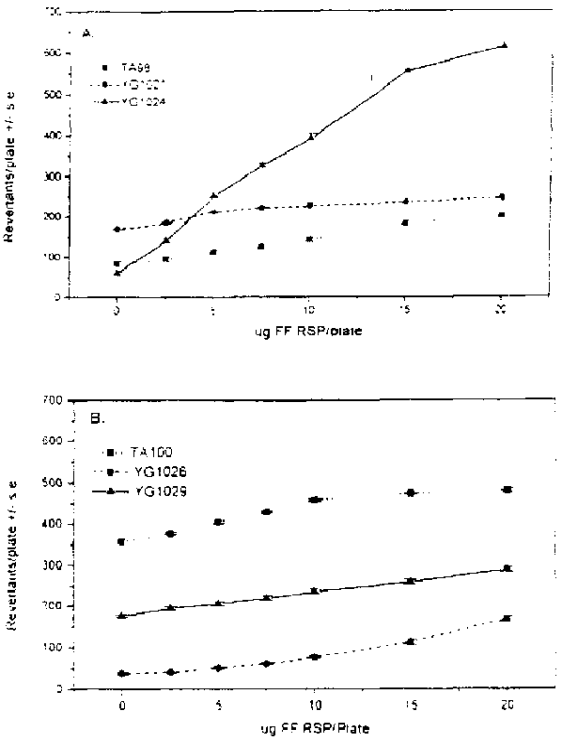

Fig. 2. Comparison of tester strains in the microsuspension assay of respirable suspended particles (RSP) of full-flavored cigarettes with 5% S9 metabolic activation. (A) Strains TA98, YG1021, and YG1024. Slope values (revertants/μg RSP) are 6.13, 4.02, and 32.13, respectively. (B) Strains TA100, YB1026, and YG1029. Slope values (revertants/μg RSP) are 10.20, 5.89, and 5.54, respectively. Results are from single assays with triplicate plates per dose.

DISCUSSION

The chemical composition and mutagenic activity of ETS generated by human smokers was measured in this study. Exposure conditions were selected to optimize a comparative study of the chemical composition and mutagenicity of concentrated ETS and to enable detection of trace amounts of ETS components. In the present study, noncigarette smoke sources of RSP were eliminated as much as possible. Background analyses showed only trace amounts of RSP prior to the initiation of smoking. The chamber was operated only in the static mode during the course of these studies. Thus, while the conditions of this study were selected to optimize the assessment of ETS, the exposure concentrations were exaggerated relative to "real-life" exposures. In this study, the average RSP concentration of the four commercially available cigarettes was 1,423 μg/m^3 while RSP from the TOB-HT cigarette was approximately 181 μg/m^3. A recent study found that actual ETS-RSP in smoking workplaces and homes were

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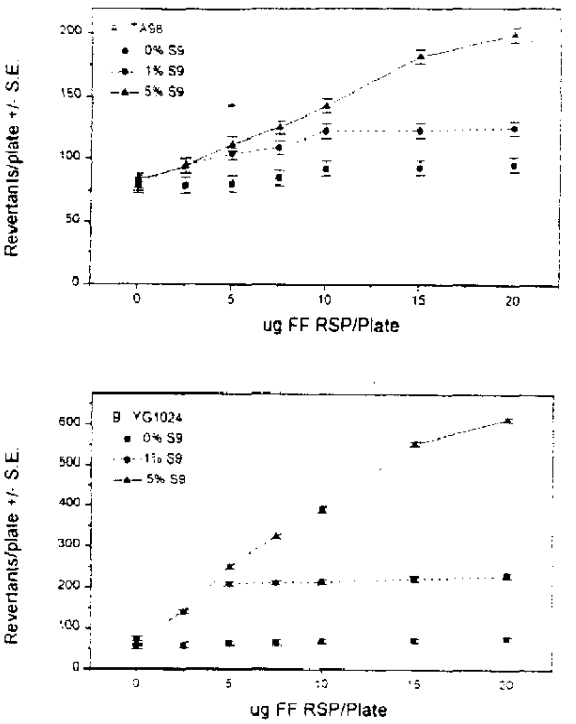

Fig. 3. Comparison of the effect of S9 concentration on the mutagenicity of respirable suspended particles (RSP) from full-flavored cigarettes in the Ames microsuspension assay. Results are from single assays with triplicate plates per dose. (**A**) Strain TA98 with 0, 1, and 5% S9. Slope values (revertants/μg RSP) are 0.97, 3.88, and 6.13, respectively. (**B**) Strain YG1024 with 0, 1, and 5% S9. Slope values (revertants/μg RSP) are 1.02, 27.05, and 32.13, respectively.

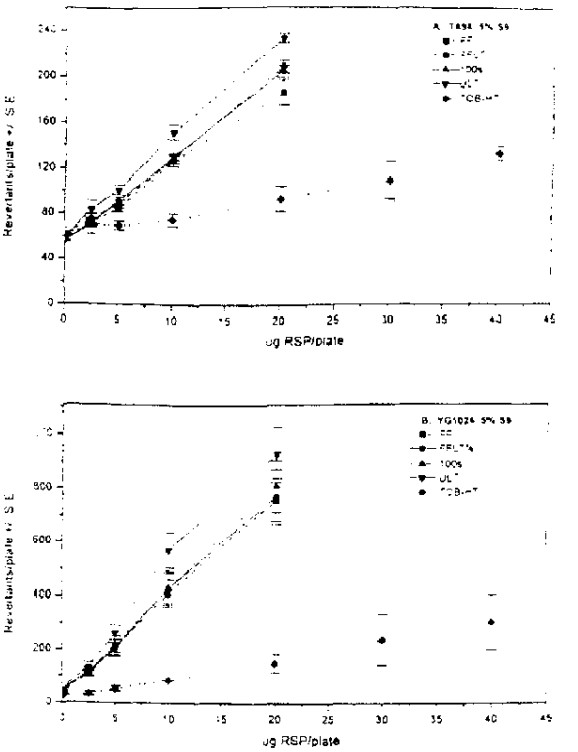

Fig. 4. Mutagenicity of respirable suspended particles (RSP) from environmental tobacco smoke of a cigarette that primarily heats tobacco and of commercially available cigarettes. Mutagenicity is expressed as revertants/plate ± standard error. TOB-HT ETS was assayed in three separate assays; ETS from commercially available cigarettes was assayed in five separate assays. Assays were conducted in the Ames microsuspension assay with 5% S9 metabolic activation. (**A**) TA98. (**B**) YG1024.

15.29 and 12.57 μg/m^3, respectively [Heavner et al., 1996]. In a review of data on indoor air published between 1980 and 1990, Holcomb reported mean differences in RSP concentrations between smoking and nonsmoking homes, offices, restaurants, and bars ranged from 21.8 to 48.3 μg/m^3 [Holcomb, 1993]. Thus, the concentrations of ETS-RSP generated in these comparative studies were 30–100 times higher than RSP commonly attributed to ETS in 'real life' environments.

Based on the results of optimization studies, YG1024 and TA98 were selected for the comparative studies. YG1024 showed greater sensitivity to the mutagens in concentrated ETS-RSP than TA98. This enhanced sensitivity suggests that these mutagens require *O*-acetyltransferase for activation [Watanabe et al., 1990]. TA98 was chosen as the second strain for two reasons: 1) TA98 was effective in detecting mutagens in the ETS RSP particulates, and 2) TA98 is more widely used than the other five strains for the assessment of the genotoxicity of tobacco smoke condensates [Sato et al., 1977; DeMarini, 1983; Steele et al., 1995; Curvall and Enzell, 1984; Brunnemann et al., 1994]. TA98 and YG1024 both detect frameshift mutagens. Reports in the literature [Sato et al., 1977; DeMarini, 1983] as well as experience in our laboratory [Doolittle et al., 1990] indicate that the frameshift strain TA98 is more responsive to the mutagens in tobacco smoke condensate than TA100, which measures basepair substitution mutagens.

The objective of this study was to compare the mutagenicity of ETS-RSP from the TOB-HT cigarette with that of commercially available tobacco-burning cigarettes. The observed reduction in mutagenic activity of ETS-RSP from the TOB-HT cigarettes relative to commercially available tobacco-burning cigarettes is a result of the actual changes in the chemical composition of ETS-RSP from the TOB-HT cigarette. The reduced mutagenicity of the chamber air (revertants/m^3) when TOB-HT cigarettes are smoked reflects both the changes in the chemical

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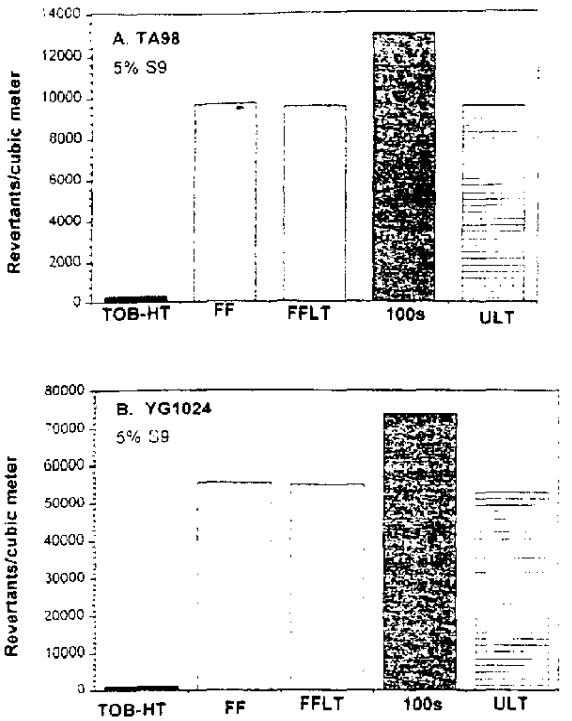

Fig. 5. Mutagenicity of respirable suspended particles (RSP) from environmental tobacco smoke of a cigarette that primarily heats tobacco and of commercially available cigarettes. Assays were conducted in the Ames microsuspension assay with 5% S9 metabolic activation. Mutagenicity is expressed as revertants per cubic meter. (A) TA98. (B) YG1024.

composition of ETS-RSP and a reduction in the amount of ETS-RSP present in air.

In conclusion, the results of our studies demonstrate that ETS-RSP from TOB-HT cigarettes is much less mutagenic than ETS-RSP from commercially available tobacco-burning cigarettes.

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